Nanotoxicology: the need for a human touch?

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Nanotoxicology: The Need for a Human Touch?

Mark R. Miller and Craig A. Poland*

With the ever-expanding number of manufactured nanomaterials (MNMs) under development there is a vital need for nanotoxicology studies that test the potential for MNMs to cause harm to health. An extensive body of work in cell cultures and animal models is vital to understanding the physicochemical characteristics of MNMs and the biological mechanisms that underlie any detrimental actions to cells and organs. In human subjects, exposure monitoring is combined with measurement of selected health parameters in small panel studies, especially in occupational settings. However, the availability of further in vivo human data would greatly assist the risk assessment of MNMs. Here, the potential for controlled inhalation exposures of MNMs in human subjects is discussed. Controlled exposures to carbon, gold, aluminum, and zinc nanoparticles in humans have already set a precedence to demonstrate the feasibility of this approach. These studies have provided considerable insight into the potential (or not) of nanoparticles to induce inflammation, alter lung function, affect the vasculature, reach the systemic circulation, and accumulate in other organs. The need for further controlled exposures of MNMs in human volunteers - to establish no-effect limits, biological mechanisms, and provide vital data for the risk assessment of MNMs - is advocated.

1. Background

Nanotoxicology is the study of the whether particles or materials in the nano-scale (with at least one dimension of <100 nm) have the potential to cause detrimental actions to cells, organs, and organisms.[1] The field of nanotoxicology has grown exponentially over the last two decades, in parallel with development of different types of nanoparticles (**Figure 1**). Whilst a significant proportion of the nanotoxicological literature addresses manufactured nanomaterials (MNMs), the field of nanotoxicology and the application of findings addresses both engineered as well as incidental (e.g., environmental) nanoparticles. It is important to recognize that from a toxicological perspective, there is arguably little distinction between engineered and incidental nanoparticles and toxicological understanding from one can inform the other.[2] There is indeed concern that the development, use, and ultimately exposure to MNMs could pose a significant risk to humans, and such concerns are well founded. This is because one only has to look at ambient nanoparticles more generally to understand that such adverse health effects are occurring in polluted towns and cities on a massive scale (see below).

Over the last few decades, such realization of the negative impact nanoparticles can have on health has led to the availability of research funding resulting in a huge body of high-quality research. The combined efforts of material characterization, computational modelling, dosimetry, in vitro and in vivo models, -omics, exposure, and other disciplines, have advanced our understanding markedly. Large-scale projects, such as the European Commission funded project GRACIOUS,[3] as just one example, are now seeking to draw together this information into a science-based framework that supports effective risk assessment.

However, the vast labyrinth of complex data that has been generated by nanotoxicological research also presents something of challenge in identifying what the next steps will be to make significant advances in the field. In their 2015 review,[4] Hussain et al. postulate that nanotoxicology has reached a "crossroads" whereby researchers need to build on past achievements to negotiate the challenges that persistently frustrate nanotoxicology research. Key challenges include: rapid pace of innovation of new MNMs outstripping the rate of conventional safety testing; uncertainty regarding possible exposure scenarios, dosimetry, and toxicokinetics; complexities in the interactions between nanoparticle physicochemical properties and cellular biology; the dynamic nature of nanoparticle coronas in biological systems; practicalities in handling nanoparticles and overcoming assay interference; the limited predictivity of current models (both in vitro and in vivo for human health effects); and many others.[2,4–17]

2. The Value of Human Data

One additional barrier for MNM research is the paucity of human data that combines both metrics of exposure with

Dr. M. R. Miller
University/BHF Centre for Cardiovascular Science
University of Edinburgh
47 Little France Crescent, Edinburgh EH16 4TJ, UK
E-mail: mark.miller@ed.ac.uk
Dr. C. A. Poland
Centre for Inflammation Research
University of Edinburgh
Edinburgh, UK

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relevant health parameters. While there is a reasonable number of "real-world" studies of nanoparticle exposure in occupational settings (see below) and good mechanistic data from cultured human cell lines, there are no large-scale epidemiological studies of MNM exposure. This reflects the relative infancy of the nanotechnology industry, meaning large-scale production (>100 tons per annum) of MNMs and commercial/public use for many forms of MNM has not yet occurred. This is very different to the scenario for particulate matter in air pollution, which has received considerable attention over the last few decades. Indeed, air quality has been pushed to prominence on the political agenda in most nations across the world. This attention has stemmed from staggering estimates of premature mortality attributed to ambient $PM_{2.5}$ (particulate matter with a diameter of 2.5 µm or less), which is estimated to be in the magnitude of several million early deaths globally every year.[18,19] Additionally, there is now increasing recognition that air pollution has detrimental effects on almost all major organs of the body, and there now exists an overwhelming body of epidemiological evidence linking exposure to particulate matter with many causes of morbidity and mortality.[20,21] Traffic-derived sources are of especial concern, due (at least in part) to the high proportion of combustion-derived nanoparticles in vehicle exhaust.[22]

Research into the health effects of air pollution is a prime example of the value of epidemiological studies in informing our understanding of the adverse impact of a certain exposure on health, and, therein, to assess current and future risk. In the case of MNMs, the goal should be to prospectively prevent ill health and so substantial effort is required to confirm negative results are attributed to effective control measures. For statistical power and assessment of potential confounding variables, epidemiological studies require large cohorts to detect relationships between exposures and specific diseases. While the nanotechnology sector is a major global industry, the general public's exposure to MNMs is limited at present (although, there is always concern that there could be overlooked exposures which have not immediately manifested as health concerns due to the prolonged asymptomatic development of disease).

Mark R. Miller is a Senior Research Scientist in the Centre for Cardiovascular Science at the University of Edinburgh, United Kingdom, funded by the British Heart Foundation. His research investigates the biological pathways by which the particles in air pollution promote cardiovascular disease. He also has an interest in the potential for manufactured nanoparticles to impair cardiovascular function. He is a member of the World Heart Federation Air Pollution Expert Group and an Expert Member of COMEAP, the UK governmental advisory Committee on the Medical Effects of Air Pollution.

Craig A. Poland is a Senior Research Fellow at the University of Edinburgh's Centre for Inflammation Research. He has worked in the field of respiratory health and, in particular, the effects of inhaled particles on the body for the last 16 years. His research interests include understanding the physicochemical and dosimetric drivers behind particle-mediated biological effects and disease outcomes. He also works on the incorporation of hazard and exposure data to inform evidence-based risk characterization and effective prevention of occupational disease. He has published widely in the field of particle and nanotoxicology.

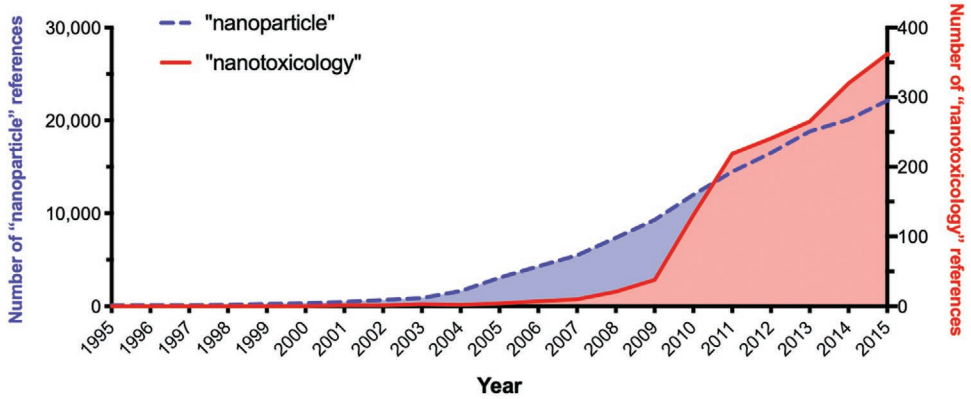

Figure 1. Increasing scientific research on nanotoxicology over the last 20 years. Number of references per year (noncumulative) based on a PubMed (https://www.ncbi.nlm.nih.gov/pubmed/) search for "nanoparticle" (blue dotted line) and "nanotoxicology" (red solid line) without further limits applied. The graph highlights that nanotoxicological research has gathered pace over recent years, reflecting the need for toxicological assessment of nanoparticles in different settings, including environmental nanoparticles in air pollution, unintentional exposure to manufactured nanomaterials (MNMs) (e.g., accidental/occupational exposure), and intentional exposure to MNMs (e.g., in nanomedicine). Use of the broad term "nanoparticle" was used to capture all three of these scenarios.

In the absence of large cohorts of epidemiological data, investigation of the biological effects of MNMs in human subjects would add a hugely valuable perspective to the wealth of mechanistic research in this field and provide an immediate reference point for risk assessment. To this end, there have been various published studies on human exposure to MNMs addressing both incidental and deliberate exposures. Incidental exposure includes that resulting from work activities for a (currently) limited number of MNMs being produced at commercial volumes. As a body of literature, they can provide important insight into potential occupational health effects resulting from repeated exposures, although such studies can suffer from the same issues of limited exposure characterization as well as complex and mixed exposure patterns. Deliberate, controlled exposures of nanoparticles as part of well-designed studies would mitigate against confounding factors found in occupational investigations. Here, we provide an overview of existing evidence for both incidental and controlled exposures to MNMs in human subjects.

3. Incidental Occupational Exposures

While epidemiological studies with large cohorts are lacking, there are a number of small-scale observational studies of MNM exposure and health, especially in occupational settings. A review of occupational exposure to nanoparticles is beyond the scope here; however, we wish to mention a few examples of workplace exposure to noncombustion nanoparticles where health parameters have been measured (see also refs. [23,24]). The examples below focus on inhalation exposure, and the reader is referred elsewhere for other routes of exposure such as oral[25,26] and dermal.[27,28]

Several studies have made use of factory floor workers in industries producing or packaging MNMs. A study of thirteen factories in Taiwan handling a variety of MNMs (including gold, silver, iron oxide, titanium dioxide, silicon dioxide, and carbon nanotubes) found that exposed workers exhibited reductions in lung function. In addition, they found an increase in Clara cell protein 16 (a blood marker of airway damage), as well as small changes in blood antioxidant and adhesion marker levels, compared to workers at the same factories with a lower risk of exposure.[29] In another study,[30] even though factory floor workers who had handled MNMs (various particles including polystyrene, zinc-cadmium, hydroxyapatite, carbon nanotubes, copper) did not appear to have a (measurable) greater level of exposure in their day-to-day work, they exhibited higher levels of several blood cytokines compared to workers who had never handled these materials. A number of other comparisons were nonsignificant or lacked clarity, perhaps reflecting the diversity of exposure dose to multiple materials.

The archetypal carbon nanoparticle, carbon black (CB), is used in a number of manufacturing industries, including tyre and rubber products, electrical insulating materials, pigments, and printer toner. Workers packaging CB in China were found to have reduced lung function (by spirometry) and raised levels of blood cytokines compared to a control group from a water treatment plant in the same city.[31] The investigators state that workers wore respiratory protection although the level of compliance was not clear. Cross-sectional studies in Iran[32] and Nigeria[33] reported similar findings, whereas others in the USA[34] and Germany[35] did not find significant associations between CB exposure and pulmonary function. Two studies have investigated carbon nanotube exposure in an occupational setting, finding greater levels of oxidative stress (blood malondialdehyde) in manufacturing workers compared to office workers in the same factory.[36] Shvedova et al., demonstrated differential profiles in mRNA and noncoding-RNA (especially those linked to the cell cycle, and possibly cardiorespiratory disease) in workers exposed to CNTs, compared to nonhandling workers.[37] Further studies are needed to address biological effects to different fiber dimensions and bio-durability of fibers.

Titanium dioxide nanoparticles are one of the most widely used MNMs, for applications that include white pigments, sunprotection cream, and food additives. A cross-sectional study of packaging workers in TiO_2 nanoparticle manufacturing plants in China, found that TiO_2 exposure was associated with impaired lung function and blood markers of oxidative stress and inflammation, and potential predictors of cardiovascular disease.[38] Similarly, markers of oxidative stress were increased in the exhaled breath condensate of office workers following a visit to the factory floor of a TiO_2 factory.[39,40]

Other metal-rich nanoparticles have been a subject of concern due, among other things, to their potential to induce health effects through oxidative stress. It has been recognized that metal welders have a higher relative risk of cardiovascular morbidity and mortality.[41,42] Ellingsen et al. made a comparison of 72 welders with suitable referents from similar occupations. Welders were estimated to have exposures of 8.1 mg m^{-3} during their working day, and accordingly were found to have higher levels of inflammatory (TNF-alpha) and pro-thrombotic biomarkers (P-selectin, CD40L, pro-thrombin, D-dimers) in the blood.[43] Similarly, volunteers that had been asked to spend several hours in settings with exposure to welding fumes exhibited a variety of inflammatory responses in the lung and blood.[24] Other occupational exposures of metal particles, such as steel plant or iron foundry workers, or occupations with exposure to particulates rich in aluminum or manganese, have shown indications of depressed anti-inflammatory respiratory defences, and possibly even neurological symptoms.[24,44]

While these occupational studies provide a useful insight into selected human populations, often there are as many negative findings as positive ones, and this may reflect study limitations or confounding variables, for example, lack of an optimum comparative control group, the mixture of nanoparticles the person is exposed to, poor characterization of exposure (time and concentration) between individuals, the need to stratify for smoking status, and occupational confounders such as high temperatures, strenuous activity, etc.

4. Nanomedicines

Nanoparticle exposure is not only an occupational issue, nor is exposure only incidental/accidental. Due to the unique properties of nanoparticles, MNMs offer many opportunities to provide benefits to a wide range of sectors. One example with immediate relevance to human health, is the development

of MNMs for purposeful administration to people for drug delivery or diagnostic agents in medical imaging.[45] For example, ultra-small paramagnetic iron oxide nanoparticles (SPIONs; with a diameter between 10–40 nm) have been tested clinically as potential alternatives to contrast agents such as gadolinium for magnetic resonance imaging (MRI). Using this imaging modality, SPIONs are being considered for uses that include cell tracking, identification of cancerous growths or visualization of cardiac blood vessels to assess coronary artery disease. The advantage of SPIONs is that, after injection, these particles localize to areas of inflammation and, therefore, could reveal tissues that are actively developing diseases, or trigger points in disease pathophysiology such as the inflamed "unstable" atherosclerotic plaques in blood vessels that could trigger a cardiovascular event such as a heart attack or stroke. As an example, Richards et al. demonstrated that it was possible to label peripheral blood monocytes with SPIONs without overtly activating the cells, allowing them to be re-administered to the volunteer by intramuscular injection.[46] The investigators demonstrated that after injection the SPION-laden cells relocated to subcutaneous areas that had been experimentally inflamed. The group have since gone on to test the feasibility and value of SPIONs in patients with myocardial ischaemia[47] and abdominal aortic aneurysm.[48] Despite the high concentrations of intravenous solutions administered, these agents appear to have good biocompatibility and seemingly little toxicity. However, the full extent of the cellular effects of different SPION suspensions after uptake by inflammatory cells (or other cell types), and potential to induce complement hypersensitivity reactions,[49] deserves further attention. This is especially true after chemical modification of the particles, for example, to increase stability, improve biokinetics, or influence the cellular target of the SPION.

In terms of nanotherapeutics, these must go through the same regulatory approval process as any new medicinal product or medical device (nanomaterials may fall into either category dependent on the mode of action). Whilst the specific approvals process differs between regulatory bodies, the process understandably requires a high degree of testing to ensure safety and efficacy. Such testing typical requires a combination of in vivo studies as well as phased clinical trials in humans; a combination not required in other regulatory environments. It is applications that have much lower testing requirements or fall out with current legislation that raise concerns about possible health effects. The rigorous testing required before new nanotherapeutics can progress to market is welcome and will allow the unique benefits of these materials to be fully realized.

5. Controlled Exposures in Human Volunteers

A study approach that has been employed with great success in the field of air pollution is that of "controlled exposure" in human volunteers (**Figure 2**). In these studies, volunteers are asked to inhale a specified test material/pollutant at a carefully regulated concentration in a "controlled" setting (e.g., in an exposure chamber or through a facemask), with ready access to clinical experience and facilities for measurement of health parameters and as a safeguard for unforeseen adverse events. This approach has a number of advantages such as the "exposure" being administered by a physiologically relevant route (i.e., inhalation, with breathing rate often regulated by use of mild/moderate exercise on a stationary bicycle or treadmill). Exposures can be tightly controlled to ensure they are identical between subjects with specific materials/pollutants administered in isolation (e.g., gases can be separated from particles, or particles can be size-segregated) to determine causative constituents of complex exposure sources. Many of the confounders of epidemiological studies can be removed or controlled, such as exposure to vehicle exhaust without traffic noise, or avoiding uncertain fluctuations in stress from driving in traffic, etc. Repeated visits (with treatments randomized between volunteers) can also be used to account for other confounding variables and allow volunteers to act as their own controls (e.g., one visit with exposure to the pollutant, a second visit uses filtered air exposure with the same conditions, with exposure type randomized). Furthermore, a wider range of health parameters can be measured that may not be practical in real world settings or gathered during more routine occupational health monitoring. Specific patient subgroups with defined health conditions can also be recruited to participate in exposures under clinical supervision to further understand effects in sensitive groups.

Controlled exposure studies have been used to assess the biological actions of environmental nanoparticles, especially combustion-derived nanoparticles in vehicle exhaust. The potential health effects of diesel exhaust (DE) have been explored using 2 h exposures to diesel exhaust diluted to levels that could be encountered on very heavily congested roads (usually 100–300 μg particulate m^{-3}). Several groups have used this approach to demonstrate the potential for DE to induce a range of health effects. These include localized effects such

Figure 2. Controlled inhalation exposures in human volunteers. Left and middle panel: Exposure chamber for diesel exhaust exposures in Umea University, Sweden. Images courtesy of Prof Thomas Sandstrom and Prof Anders Blomberg. Right panel: volunteer in the University of Edinburgh Clinical Research Facility, where vascular responses are being measured by forearm plethysmography. Image courtesy of Prof Nicholas Mills.

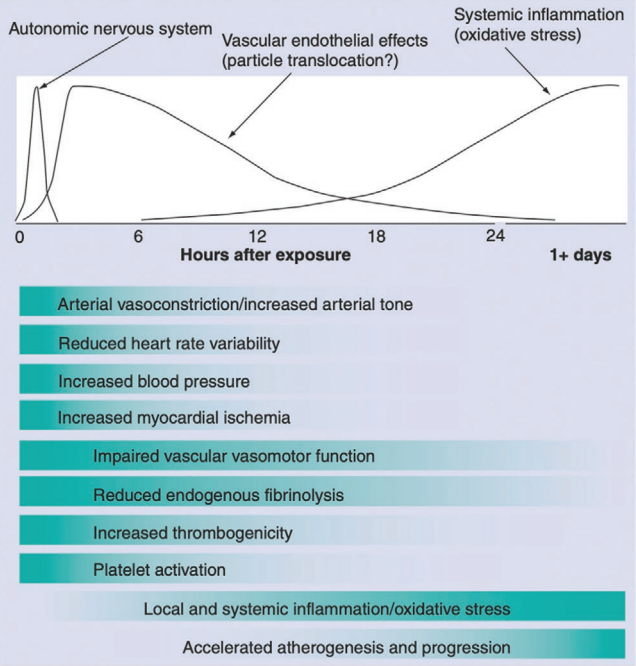

Figure 3. Controlled exposure to diesel exhaust impairs cardiovascular function. Schematic of the time course of the multiple cardiovascular effects of acute exposure to diesel exhaust. Reproduced with permission.[92] Copyright 2012, The Author, published by Future Medicine LTD (part of Future Science Group).

as bronchoconstriction and increased airway resistance,[23,50,51] pulmonary inflammation,[23,52–59] and oxidative stress,[50,55,60] DNA methylation in bronchial biopsies or blood cells,[61,62] and exacerbation of allergic responses[63] as well as reductions in parameters related to exercise capacity.[64] Systemic effects have also been analyzed such as inflammation[52,65–67] and oxidative stress,[68–70] increased levels of circulating oxidized lipoprotein receptors,[71] increased blood pressure,[72] impaired vascular function (arterial contraction),[73,74] reduced vasodilator responses,[75–79] increased arterial stiffening,[80] promotion of blood clotting,[69,81] and increased cardiac ischaemia[82] (**Figure 3**). Experiments that removed the particulate fraction of DE using experimental filtering or exhaust particle traps[83,84] demonstrated that it was the particulate constituents of DE that drive cardiovascular impairments. This approach has subsequently been used to investigate other environmentally relevant particles such as wood-smoke particles (e.g., refs. [85–88]) and exhaust from biodiesel fuels.[89–91] These approaches have been invaluable in demonstrating the targets and mechanisms by which combustion-derived nanoparticles promote cardiorespiratory disease.

There are a few isolated studies that have performed controlled exposure to MNMs in human volunteers. Our group investigated which constituents of DE were responsible for cardiovascular impairment using a four-visit study design with exposures to i) DE, ii) filtered air, iii) filtered-DE, and iv) Palas spark-generated carbon particles (**Figure 4**).[84] The latter group was used to assess the effects of a "clean" carbon nanoparticle to compare to that of the more chemically complex diesel exhaust particle (which can be viewed as a carbon core with a complex surface chemistry containing transition metals and a vast array of organic carbon species). Due to the smaller size of the Palas generated particles, identical mass concentrations could not be reached (70 μg m^{-3} for Palas; 348 μg m^{-3} for DE), however, the Palas exposure contained double the particle numbers (3 865 000 vs 1 198 000 for DE) and presumably a greater surface area dose. While DE impaired vascular function, neither the Palas particles nor the DE devoid of particles, alter vascular responses. It was concluded that the complex surface constituents of DE particles were responsible for driving the vascular effects of DE in humans. These findings have parallels with that of an earlier human exposure study using lower concentrations of carbon nanoparticles (10–50 μg m^{-3}; 30–40 nm count median diameter) which also found only subtle or no effects on lung function, exhaled nitric oxide or sputum biomarkers.[93] From the viewpoint of manufactured nanoparticles, the result is intriguing and will help inform the debate as to whether "pure" carbon nanoparticles, such as carbon black pose a risk to health. While they are generally not considered as high-toxicity materials, rodent studies have clearly shown that nanosized carbon particles are far from completely inactive, being able to instigate both oxidative stress and inflammation.[94–97] However, such effects are often seen at very high doses associated with lung overload with considerable debate as to whether or not such effects are species specific and therefore of relevance to humans.[96]

Controlled human exposures of MNMs have also been used to address the possibility of particle translocation from the lung to the systemic circulation. While there is now considerable evidence in animals that inhaled nanoparticles cross into the blood to reach other organs (see [98]), prior to 2006 there was only an isolated study investigating whether a similar process occurred in human subjects. Nemmar et al. performed inhalation of Technegas in healthy volunteers. Technegas contains ^{99m}Tc-labeled carbon particles of 5–10 nm primary diameter, that can be detected at very low concentrations by using a gamma counter.[99] The investigators detected radioactivity in the blood of volunteers a minute after inhalation, with levels reaching a maximum in the blood at 10–20 min and continuing to increase in the liver over 60 min. However, subsequent studies by separate groups[100,101] failed to replicate these findings. While radioactivity could be detected in the blood of Technegas-exposed subjects, thin layer chromatography suggested that the blood signal arose from the soluble pertechnetate label rather than particulates,[100] and thus re-opened the question as to whether particles per se can translocate to the blood in man.

Recently, our group reassessed particle translocation in human subjects using gold nanoparticles (**Figure 5**).[102] Gold offers a range of advantages over other model particles: i) the substance is relatively inert and safe for human administration, ii) gold nanoparticles can be synthesized in a range of sizes, including those within the primary size range of DE, iii) the body should contain negligible levels of gold basally (unlike carbon), and iv) a number of extremely sensitive techniques are available to detect gold at the low levels occurring in systemic organs after translocation. Following inhalation of spark-generated gold nanoparticles (4 nm primary size, 19 nm aerodynamic diameter) in healthy volunteers (2 h at 116 μg m^{-3} during intermittent moderate exercise), gold could be measured

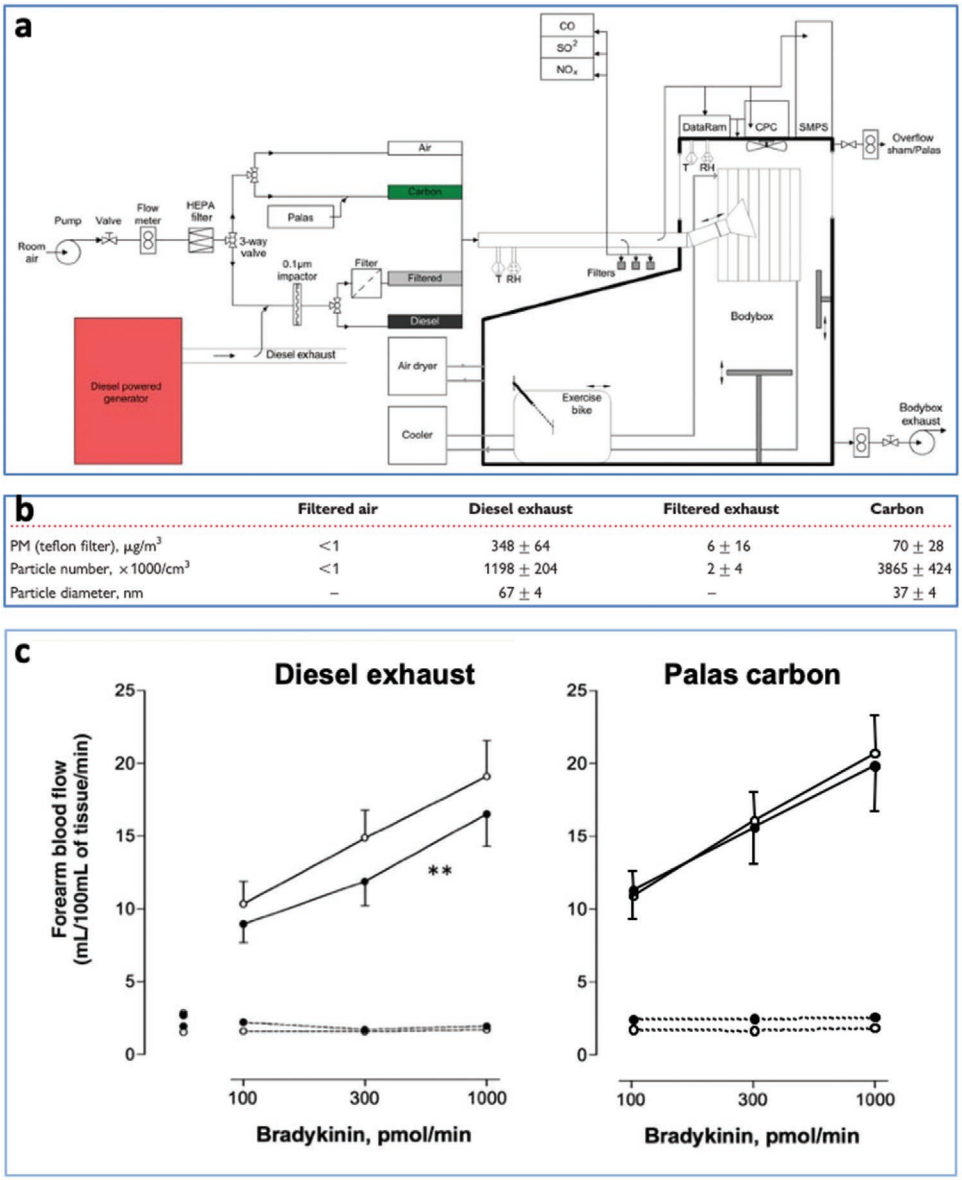

Figure 4. Controlled exposure of diesel exhaust compared to "clean" Palas carbon nanoparticles. a) Schematic of exposure chamber for a comparison of exposures of clean air, diesel exhaust, filtered diesel exhaust, or Palas carbon nanoparticles. b) Characteristics of four exposures. c) Diesel exhaust, but not Palas particles impaired forearm vascular responses (increasing forearm blood flow with infusion of the vasodilator bradykinin). Schematic and data in (a) and (b) and graph in (c) for diesel exhaust: Reproduced with permission.[84] Copyright 2011,The Authors, Published by European Society of Cardiology. Graph of Palas data: previously unpublished.

in the blood within the 24 h after exposure. Interestingly, gold was still found in the blood of these volunteers three months after the exposure. Heat-fusing of gold nanoparticles allowed larger particles to be synthesized (34 nm primary diameter, 52 nm aerodynamic diameter). On inhalation, the smaller 4 nm diameter gold nanoparticles more readily entered the blood and was found in the urine, compared the larger 34 nm particles. Lastly, patients were recruited that had a history of cerebral ischaemia (stroke) due to the build-up of inflamed atherosclerotic plaques in their carotid arteries. A small number of volunteers were willing to inhaled gold nanoparticles (4 nm) on the day prior to their surgery to remove the diseased plaque from the arteries. Raman spectromicroscopy was used to demonstrate the accumulation of gold in the diseased tissue that was removed. The ramifications of these findings have been discussed previously[98,102] but, if nothing else, these studies demonstrate that it is possible to perform exposures to manufactured nanomaterials in both healthy volunteers and patient groups, if using low-toxicity materials in carefully controlled and ethically performed clinical studies.

There are only a very small number of exposure studies to MNMs in controlled (nonoccupational) settings. Firstly, Sikkeland et al. carried out 2 h inhalation to aluminum oxide particles (≈4 mg m^{-3}, 3 nm primary diameter) in 15 healthy male students.[103] The exposure caused a modest increase in levels of interleukin-8 in the volunteers' sputum, without significant

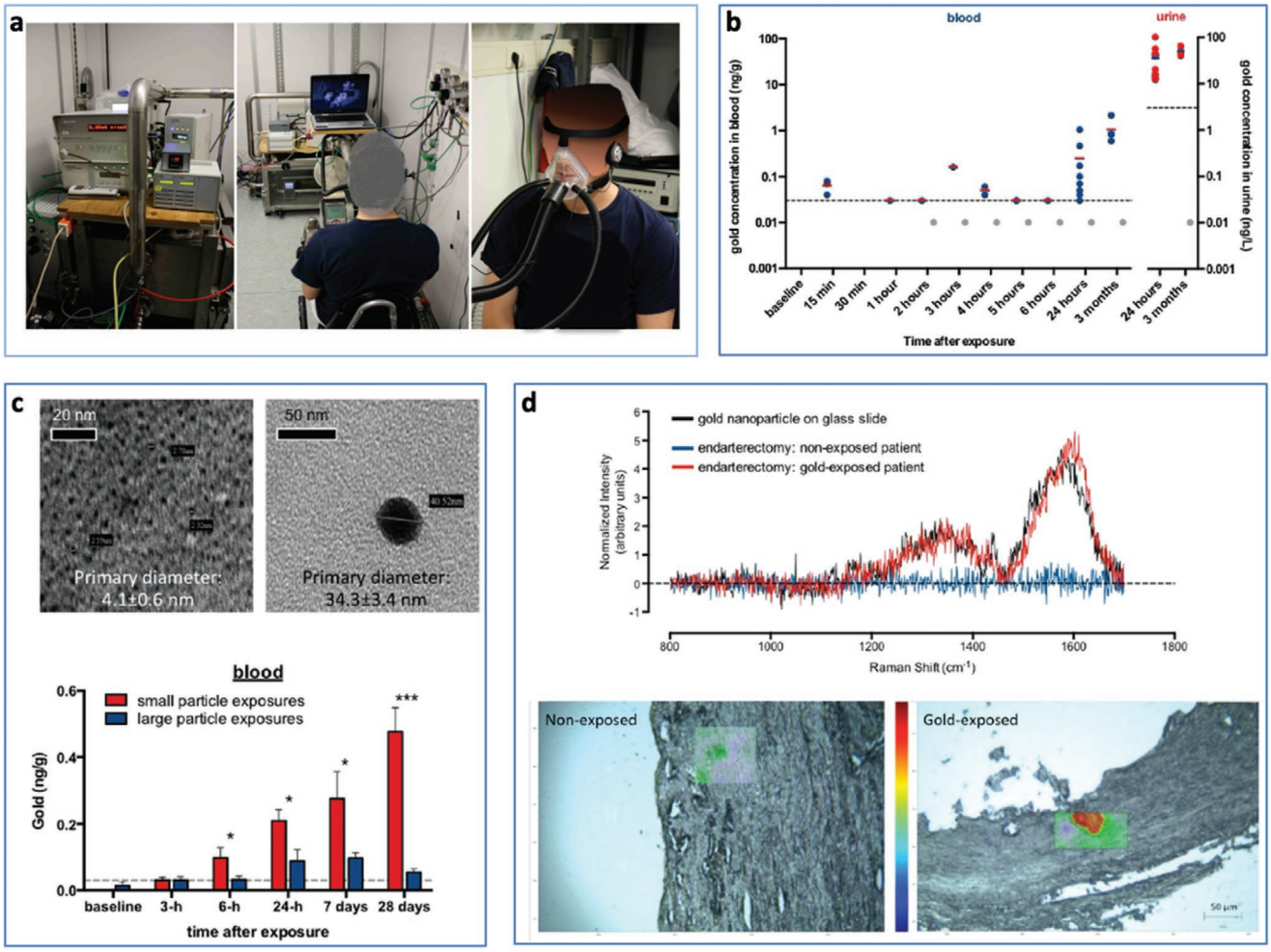

Figure 5. Controlled exposure of gold nanoparticles for the exploration of particle translocation. a) Inhalation of gold nanoparticles through a facemask by an exercising volunteer. b) Detection of gold in the blood and urine of participants by inductively coupled plasma mass spectrometry. c) Greater translocation of small (4 nm) versus large (34 nm) nanoparticles. d) Detection of gold particles (yellow, orange, and red colors; Raman microspectroscopy) in the atherosclerotic plaque removed from stroke patients following carotid endarterectomy. Reproduced under the terms of the ACS Editors' Choice with CC-BY license (https://pubs.acs.org/page/policy/authorchoice_ccby_termsofuse.html).[102] Copyright 2017, American Chemical Society.

changes in neutrophils or levels of matrix metalloproteinase (MMP)-8 compared to the sham exposure (clean air). Secondly, exposure to nanoparticle-rich welding fumes (1 mg m^{-3} intermittently for 3 s every 20 min for 5.5 h) in a chamber setting led to mild increases in IL-6 in the nasal lavages of welders without lower airways symptoms, whereas there were no significant changes in lung function or blood biomarkers.[104] To the best of our knowledge, we are aware of only a two sets of experiments that have performed inhalation exposures to a MNM of potentially greater toxicity (excluding combustion-derived particle studies), both using zinc oxide nanoparticles.[105–108] Beckett et al. carried out controlled exposure to 0.5 mg m^{-3} ZnO nanoparticles (≈40 nm count median diameter) in healthy volunteers, and found no effect on subject-reported symptoms, cardioelectrophysiology (heart rate variability) or blood markers of inflammation or coagulation.[105] In contrast, Monsé et al. performed controlled exposure to higher doses of ZnO nanoparticles (**Figure 6**). Sixteen healthy volunteers inhaled ZnO nanoparticles (≈10 nm primary diameter) for 4 h at a dose of 0.5, 1 or 2 mg m^{-3}. Inhalation of ZnO caused a concentration-dependent increase in systemic inflammation (C-reactive protein, serum amyloid-A, and blood neutrophils) and the highest dose caused mild flu-like symptoms in some volunteers.[107] The exposure also caused airway inflammation and symptoms of irritation, although the effects were not concentration-dependent.[108] There was no indication of oxidative stress ($F_{2\alpha}$-isoprostane levels) in the sputum, but matrix metalloproteinase-9 (MMP-9) levels were increased. The findings provided valuable information to inform the setting of occupational levels of zinc nanoparticles. Exposure limits for ZnO are set at 5–10 mg m^{-3} in many countries,[109] yet these controlled exposure studies suggested that a No Observed Adverse Effect Level (NOAELs) is likely to be a magnitude smaller than this in the case of the nanosized materials. It should be noted that factors such as level of activity of participants, particle size, and, importantly, solubility of the metal oxide nanoparticles, is likely to have an impact on the biological response and subsequent NOAELs.

Finally, in the interests of completeness, we should also acknowledge that there are a number of controlled human

a

ID	Temperature				CRP				SAA				Neutrophils [%]			
	ZnO-Conc. [mg/m³]				ZnO-Conc. [mg/m³]				ZnO-Conc. [mg/m³]				ZnO-Conc. [mg/m³]			
	0	0.5	1.0	2.0	0	0.5	1.0	2.0	0	0.5	1.0	2.0	0	0.5	1.0	2.0
1	3	1	2	4	1	2	3	4	1	2	3	4	1	2	3	4
2	1	2	3	4	1	2	3	4	1	2	3	4	2	1	3	4
3	4	1	2	3	4	1	2	3	4	1	2	3	4	3	1	2
4	1	4	3	2	2	1	3	4	2	1	3	4	4	1	2	3
5	1	2	4	3	1	2	3	4	2	1	3	4	1	2	3	4
6	1	3	2	4	1	1	3	4	2	1	3	4	1	3	2	4
7	1	2	3	4	1	2	3	4	2	1	3	4	1	2	3	4
8	3	4	1	1	1	2	3	4	4	1	2	3	1	2	3	4
9	3	1	4	2	1	2	3	4	1	2	3	4	1	4	2	3
10	1	1	3	4	2	1	3	4	2	1	3	4	1	1	3	4
11	2	1	3	4	1	2	3	4	1	2	3	4	1	2	3	4
12	4	1	2	3	1	2	3	4	2	1	3	4	1	2	2	4
13	4	1	4	4	1	3	2	4	3	1	2	4	1	3	2	3
14	1	3	1	4	1	2	3	4	1	2	3	4	1	2	3	4
15	2	1	4	3	2	1	3	4	1	2	3	4	1	2	3	4
16	2	1	3	4	2	1	3	4	1	2	3	4	1	4	3	2

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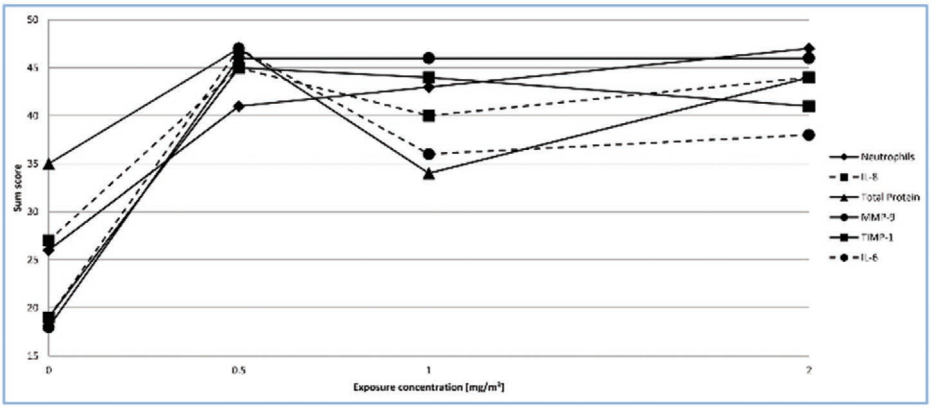

Figure 6. Controlled exposure to zinc oxide nanoparticles. a) Inhalation of ZnO nanoparticles led to a concentration-dependent increased in inflammation (CRP = C-reactive protein; SAA = serum amyloid-A). Data are presented as a ranked score from 0 (no change) to 4 (maximum change), with shaded numbers representing increases in values and unshaded numbers representing decreases. b) All tested concentrations of ZnO led to increased biomarkers of inflammation (neutrophils; IL-8 = interleukin-8; total protein; IL-6 = interleukin-6), matrix metalloproteinase (MMP-9), and tissue-inhibitors of metalloproteinase (TIMP-1) in sputum (presented as a score of ranks). Reproduced under the terms of the CC-BY Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/).[107] Copyright 2018, The Authors, published by BioMed Central (part of Springer Nature). Reproduced under the terms of the CC-BY Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/).[108] Copyright 2019, The Authors, published by BioMed Central (part of Springer Nature).

exposures to nanoparticles via the dermal route.[110–112] These studies tend to investigate TiO_2 and ZnO due to their use in sun-protection creams. One such example was that of Gulson et al.[112] who studied the dermal penetration of two different sizes (≈20 nm and >100 nm) of ZnO nanoparticles enriched with the stable isotope ^{68} Zn. The material was incorporated into sunscreen formulations and applied to the backs of human volunteers twice a day for 5 consecutive days. Little ^{68} Zn was detected in blood and urine in the subsequent 6 days suggesting that the overwhelming majority of applied nanoparticles were not absorbed. The use of human volunteers was valuable to overcome interspecies differences in skin properties, such as thickness and follicular density, and the setting in which the study was conducted (sunscreens were applied to humans undergoing normal activities at a beach) further demonstrates the utility of human studies to address complex scenarios that cannot be fully replicated in animal models.

6. Limitations of Human Controlled Exposure Studies

Controlled human studies provide the opportunity to establish a better understanding of the effects of MNM on human health without waiting for widespread exposures to occur. They can be seen as a proactive, rather than reactive, approach that helps mitigate some of the uncertainty caused by the paucity of more comprehensive epidemiological data. Furthermore, they have clear benefits over the use nontarget species models such as rodents that could exhibit different physiological responses and sensitivities to MNMs. Additionally, they can help extrapolate our understanding of the data outputs from the in vivo and, possibly, in vitro evidence base for more effective hazard and risk assessment. However, controlled human exposures, like any model, have their own limitations that need to be understood in order to make the most of such a powerful tool and avoid misinterpretation of findings.

One limitation is in understanding the nature of a detected response in terms of adversity and the wider disease pathway. Put simply, it is unethical to harm someone and, therefore, human-exposure studies will always be limited in terms of the severity and duration of effect. Quite rightly, ethical approval is unlikely to be forthcoming for exposure of participants to potentially hazardous materials, for example, certain forms of long carbon nanotubes known to cause mesothelioma in rodents.[113] Furthermore, the levels of exposure allowed are likely to be limited to levels associated with minimal health risks and, subsequently, necessitate the use of sensitive techniques to measure more subtle changes in biomarkers or health parameters. Additionally, the majority of human controlled exposure studies use healthy volunteers (although not all, e.g., refs. [23,82]) due to logistical difficulties in enrolling patients and the inherent risks of using susceptible individuals. Many patients will usually be taking medication, which adds an additional factor of uncertainty and may even directly the inhibit the pathways of nanoparticle action that are under investigation. Cessation of medication is rarely ethically acceptable. In this regard, cellular and animal models of disease offer a valuable tool to study the actions of nanoparticles in diseased processes.

Another important factor is that controlled exposures are inevitably acute in nature. It is both impractical and ethically questionable to repeatedly expose participants to test substances over chronic or even sub-acute periods. Instead, exposures tend to be isolated and for relatively short durations (e.g., 1 h to 6 h) with sufficient recovery or washout periods (usually at least two weeks, ideally more) between participant exposure visits. As such, controlled human exposures may not address the possible cumulative effects arising after repeated exposures, or adaptive responses to exposures. Thus, controlled human exposure studies tend to focus on early, transient effects in order to make judgments as to possible long-term disease outcomes by considering possible adverse outcome pathways within the context of the wider toxicology test model landscape.

Lastly, studies in humans have principally focused on respiratory and selected cardiovascular parameters, with the use of blood and urine biochemistry to assess effects on other organ systems. Given the growing appreciation that inhaled nanoparticles can access the systemic circulation,[114,115] and the awareness of the multi-organ effects of particles in air pollution,[20,21] it will be important to further address MNM toxicity in other organs.[116] Studies in animals and cells will be extremely valuable in this regard, due to the availability of tissues that can be harvested and the ability to clarify mechanisms in specific cell types in isolation.

7. Concluding Remarks

Continued research in nanotoxicology is essential to assess the potential risk of exposure to MNMs and ultimately establish a safe-by-design approach to the development of new MNMs to maximize the unique properties of these materials. As we enter a new decade of nanotoxicological research, it will be important to ensure that future research progresses at a rate that keeps up with the commercial development of MNMs. Given the large amount of effort, time, and funding that have already taken place, it will be important to make sure that research ensures that relevant materials are tested not only in commonly-employed foundation assays, but also newer approaches that offer greater mechanistic insight and more relevance to human exposure scenarios. It is the authors' opinion that there is a need for more studies using carefully designed controlled exposure in human subjects. While this may not be ethically feasible for high-toxicity materials, a precedent has already been set for human nanoparticle exposures in the field of air pollution and with isolated studies with inhalation of low-to-moderate toxicity MNMs. This is particularly the case for materials of high commercial interest that may proceed to use in society, and those materials where there is a greater risk of human contact through occupational or accidental exposure. Human data is not a prerequisite for risk assessment. Studies in animal models, cell cultures, and other in vitro assays have been vital to our understanding of nanoparticles in the body; generating valuable complementary data to the limited human data currently available and establishing crucial mode-of-action pathways that would not have been possible from human studies alone. Indeed, the great efforts and valuable information obtained by preclinical nanotoxicology has already played a fundamental role is establishing informative nanosafety platforms and risk assessment tools. However, we feel that the addition of controlled human exposures studies would make a vital adjunct to existing methods and significant next step in nanotoxicological research.

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Conflict of Interest

The authors declare no conflict of interest.

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